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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The analysis of acetylcholine (ACh) levels, metabolism and release, as well as the uptake of choline, were performed on segments of rat iris to investigate the mechanisms involved in the response of the iris to acute and chronic cholinesterase inhibition. At various times after the topical administration of 0.1% DFP in sesame oil to the corneal surface, the rats were decapitated and the irises were removed. Pupil size was measured and ACh levels and cholinesterase activity were determined in each iris

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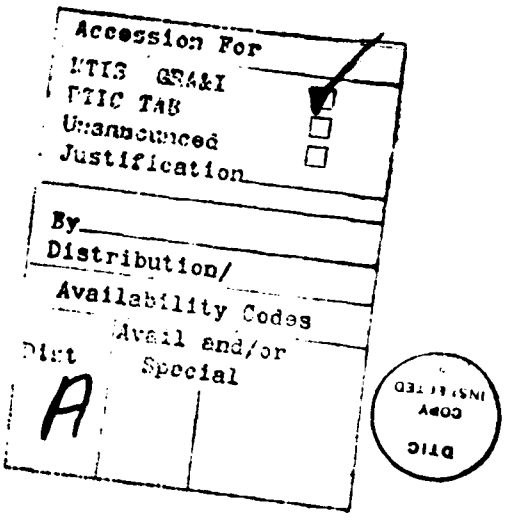
20. Abstract (continued)

following extraction of remaining free DFP with chloroform. No changes were detected 1 min. after the DFP, but by 5 min., pupil diameter was reduced by 50%, esterase activity was reduced by 90%, and ACh was increased by 60%. There were no further changes 30 min. after the DFP, but by 60 min., ACh had returned to control levels even though esterase activity was still inhibited by 90% and pupil diameter was still reduced by over 50%.

Choline is taken up by the rat iris by a low affinity process (K_{m1} = 100.6 μM) and by a high affinity active transport system (K_{m2} = 6.67 μM) that is temperature sensitive, sodium dependent, and is blocked in a dose dependent manner by hemicholinium in 10 μM or greater concentrations. Choline uptake is also reduced by millimolar concentrations of scopolamine and ouabain.

Electrical stimulation by 20 mA, 5 msec, 100 Hz nearly square waves of isolated rat iris prelabeled by incubation at 37°C in Elliott's B buffer with tritiated choline, evokes a 1- to 2-fold increase in the release of tritium over the spontaneous release during pre-stimulation baseline. Scopolamine and DFP alter the release profile with 10 nM scopolamine increasing evoked release, 1 μM scopolamine increasing spontaneous release, and 1 μM DFP reducing both spontaneous and evoked release. These results are consistent with the existence of presynaptic muscarinic receptors that control the release of ACh from cholinergic terminals in the rat iris.

Based on these results, a mechanism of action of DFP involving multiple effects on ACh metabolism is proposed.



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AFOSR Grant Number 81-0229
First-Year Activity Report

ACUTE EFFECTS OF ANTICHOLINESTERASE AGENTS ON PUPILLARY FUNCTION

LABORATORY OF NEUROPSYCHOPHARMACOLOGY
DEPARTMENT OF BIOBEHAVIORAL SCIENCES
UNIVERSITY OF CONNECTICUT

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Controlling Office: USAF Office of Scientific Research/NL
Bolling Air Force Base, DC 20332

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GRANT AFOSR-81-0229

REPORT OF 1st YEAR ACTIVITY

Prior to an investigation of the acute and chronic effects of drugs inhibiting cholinesterase on the biochemical parameters of uptake. release and metabolism in cholinergic neurons innervating the eye, the uptake of choline and the release of acetylcholine were characterized in the isolated iris of the rat. The iris contains nerve endings whose cell bodies are located in autonomic ganglia. This makes the iris a good model for the study of nerve terminal function relatively free from contamination by cell body and glial effects.

A new procedure was developed allowing to perform a multiple set of microanalyses of ACh (acetylcholine) metabolism and release, as well as of Ch (choline) uptake, in segments of single rat irises.

Methods

Biochemistry

A diagram of the utilization of a single rat iris (w.w. = 1-1.5 mg) is reported in Fig. 1. The iris is dissected out and divided under a stereomicroscope in three equal segments. Each segment is processed separately.

In the choline uptake studies, the first segment A of the irises was each preincubated in .5 ml Elliot's B buffer for 5 min at 37°C. In this portion, total radioactivity taken up is separated to account for ACh formed, ^{3}H-Ch present, and ^{3}H-phosphorylcholine (PhCh) formed.

The second segment B is utilized for the release study of tritiated ACh following incubation with ^{3}H-Ch and electrical stimulation. This part of the experiment can now be automatized to a great extent.

The third segment C is processed for the assay of endogenous levels of ACh and Ch (Goldberg and McCaman, 1973). This procedure minimizes differences due to individual variations between irises and allows for a larger number of tests to be performed in the same animal. The simultaneous measurements make it possible to estimate the rate of synthesis of ACh as well as the levels of choline and phosphorylcholine. Separation, identification and assay of each component are performed in each sample.

Pharmacology

Figure 2 shows a block diagram of the infrared video-pupillometry system used by us to continuously monitor the area of the rat pupil under different experimental conditions. Area is preferred over diameter since

area is directly proportional to the quantity of light entering the eye, regardless of pupil shape and irregularities. The major components of the video-pupillometer are:

1. The "bright pupil" infrared optical system of illumination which vertically scans the pupil producing 525 television lines every 1/30 sec. Therefore, the pupil area signal is updated 30 times/sec. The total pupil area produced by the scanner is converted to an analog voltage and displayed on the digital meter.

2. A standard closed-circuit TV camera with monitor.

3. A digital video signal processor with a digital display of the pupil area.

4. A TV monitor.

5. Chart and tape recorder.

This equipment accurately measures the pupil area of small animals in real time (30 samples/sec).

Results and Conclusions

Choline Uptake

The characteristics of the high and low affinity Ch uptake system which have been previously described by us for the developing and aging avian iris (Marchi et al., Dev. Neurosci. 3, 185, 1980 and Brain Res. 195, 423, 1980) have now been determined for the adult rat iris as well.

The uptake of choline by rat iris is linear over at least six concentrations of choline ranging from 0.1 µM to 10 µM (linear regression coefficient = 0.99). The rat iris exhibits two distinct Ch uptake systems. One component, a Na^+ dependent, temperature sensitive, high affinity system which is blocked by ouabain and hemicholinium, is most likely confined to cholinergic nerve terminals. A second component, probably localized in the iris muscle cells, is Na^+ independent and shows low affinity. The substitution of 80% Na with lithium decreased uptake by 55%.

The kinetic curves show a low affinity and high affinity uptake system with a K_{m1} = 100 µM and K_{m2} + 6.67 µM, respectively.

The effect of various drugs on choline uptake is reported in Fig. 3 as a % control. Choline uptake, as expected, could be inhibited by ouabain which is consistent with Na^+ dependent uptake, and by hemicholinium. However, the iris preparation seems to be less sensitive to hemicholinium than brain preparations as a 100 µM concentration was necessary in order

to produce a 35% inhibition of uptake. Of particular interest is the effect of DFP which at 1 mM concentration inhibits choline uptake by approximately 30%. Scopolamine (1 mM) showed a 25% inhibition effect. This effect at higher concentrations may relate to its effect on release as seen in the following experiments.

Release of Acetylcholine

Acetylcholine was released from iris preloaded by incubation with 1 μM ^{3}H-Ch by 20 mA, 5 ms, 100 Hz bipolar nearly square waves. The release is reported in Fig. 4 as a percent of the total tissue radioactivity due to ACh at each individual time. Four to six subsequential stimulations were performed starting after spontaneous release had levelled off. Released tritiated ACh and Ch were separated and counted at each point. The evoked release ratio (ERR) was calculated from the areas under the curve representing released radioactivity and compared with corresponding non-stimulated controls. Electrical stimulation evokes 1- to 2-fold increase in the release of ^{3}H-ACh over the spontaneous release during prestimulation baseline. The stimulated release is frequency dependent, tetrodotoxine sensitive and Ca^{++} dependent.

Scopolamine 10 nM increased evoked ACh release by at least 2-fold while 1 μM scopolamine increased spontaneous release only. These effects support the hypothesis of the presence in the iris terminals of a presynaptic muscarinic autoreceptor which controls release (Fig. 4).

$^{-D}$ (1 μm) reduced both the spontaneous and the evoked release by 30% (Fig. 4). The effect was stronger following the first stimulations. This concentration of DFP inhibits AChE by approximately 80% and corresponds to the concentration used in the following in vivo experiments. This increase was blocked by scopolamine.

Acute Effects of DFP on the Iris

In this series of experiments the acute effects (up to 60 min) of DFP on the pupil were studied (Fig. 5).

5 μl of a .1% solution of DFP (corresponding to 5 μg of a 5 mM concentration) was instilled in the conjunctival sac of rats and its effect on the pupil area or diameter was recorded.

1. AChE activity was inhibited by 65% at 1 min and 95% at the following times (up to 1 hr).

2. Pupil size was unchanged at 1 min but decreased by more than 50% at successive times up to 1 hour.

3. ACh levels were unchanged a 1 min, were increased by 60% at 5 and 30 min and returned to original normal levels at 60 min.

4. Choline levels were unchanged at all times, showing a tendency toward a slight increase at 30 min. In part, this reflects the low rate of hydrolysis of ACh in the presence of DFP. It is interesting to note that the pupil was still constricted at a time (60 min) when ACh had returned to baseline values. This

may be due to a prolonged effect of ACh maintaining constantly high levels at the postsynaptic site.

In conclusion, it can be said that our results are consistent with the concept of existence of presynaptic muscarinic autoreceptors that control release of ACh from the cholinergic nerve terminals in the rat iris. With regard to the mechanism of action of DFP on the iris, we propose that the drug may exert multiple effects (see Fig. 6), such as:

a. inhibition of AChE with following increase in ACh levels both intra- and extrasynaptically

b. inhibition of Ch uptake, and

c. reduction of both spontaneous and stimulated release.

Points b and c need further examination.

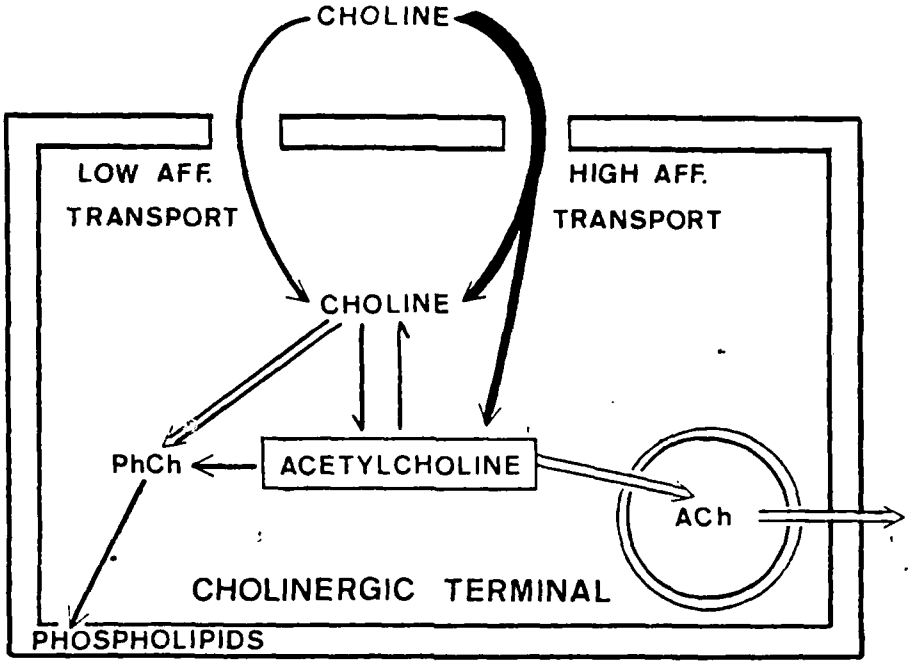
ABSTRACTS RESULTING FROM THE RESEARCH SUPPORTED BY THIS GRANT

Ann. Meeting, Soc. for Neuroscience, Minneapolis, Minn., Oct., 1982. Effects of DFP and other drugs on cholinergic nerve function in the rat iris, J. S. Richardson, T. G. Mattio, H. L. Bernstein-Goral and E. Giacobini.

European Symp. on Cholinergic Transmission Presynaptic Aspects, Strasbourg, France, May, 1982. Mechanisms of choline uptake and acetylcholine release in peripheral cholinergic synapses, E. Giacobini, J. S. Richardson and T. G. Mattio.

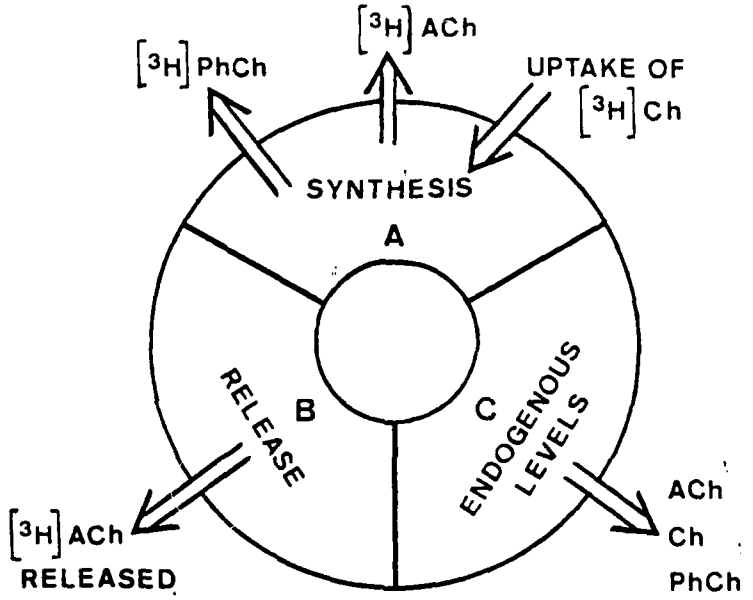
Amer. Soc. for Neurochemistry - Thirteenth Annual Meeting, Grossinger, N. Y., March, 1982. Uptake and release in cholinergic nerve terminals of the rat iris, J. S. Richardson, T. G. Mattio and E. Giacobini.

a)



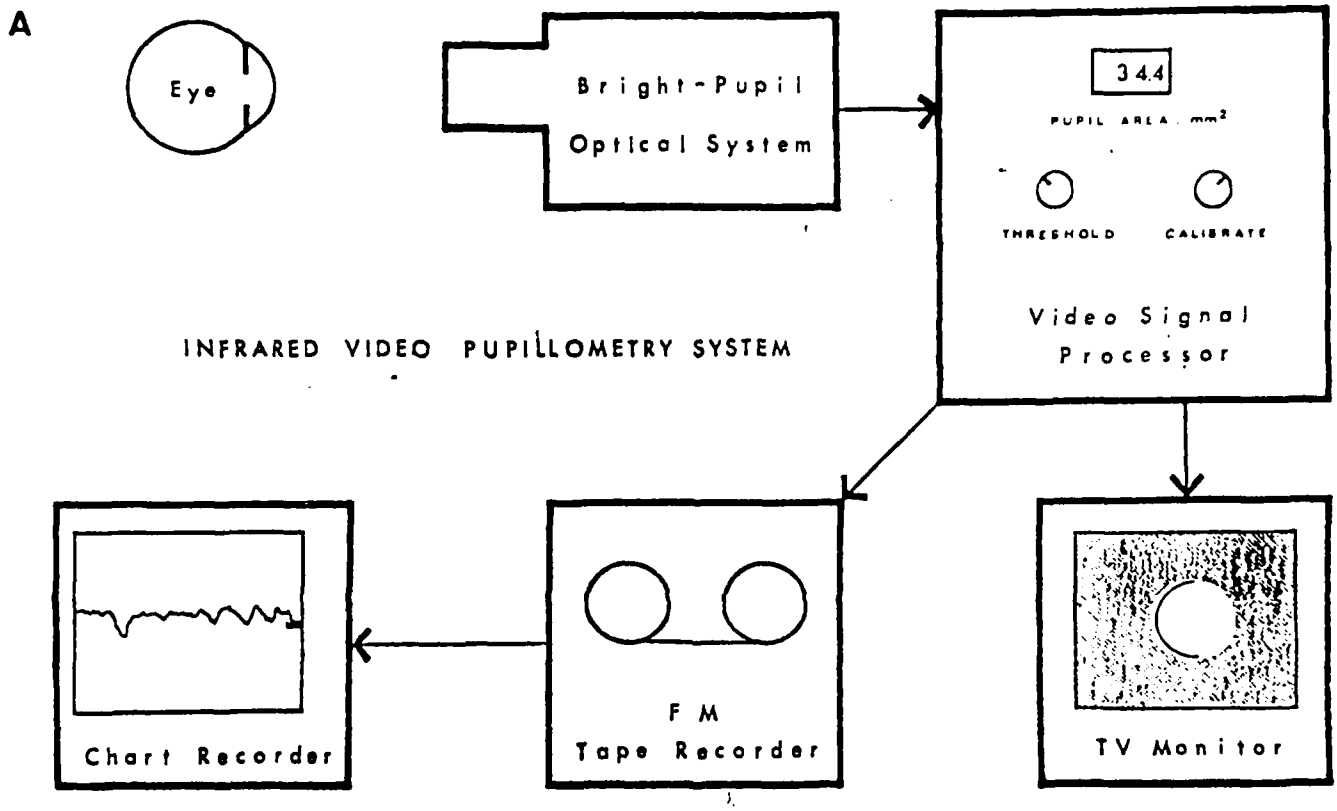
ACh = acetylcholine, PhCh = phosphorylcholine, Ch = choline

b)



IRIS PREPARATION

Figure 1


A
Eye
Bright-Pupil
Optical System
344
PUPIL AREA, mm²
THRESHOLD
CALIBRATE
Video Signal
Processor
INFRARED VIDEO PUPILLOMETRY SYSTEM
Chart Recorder
F M
Tape Recorder
TV Monitor

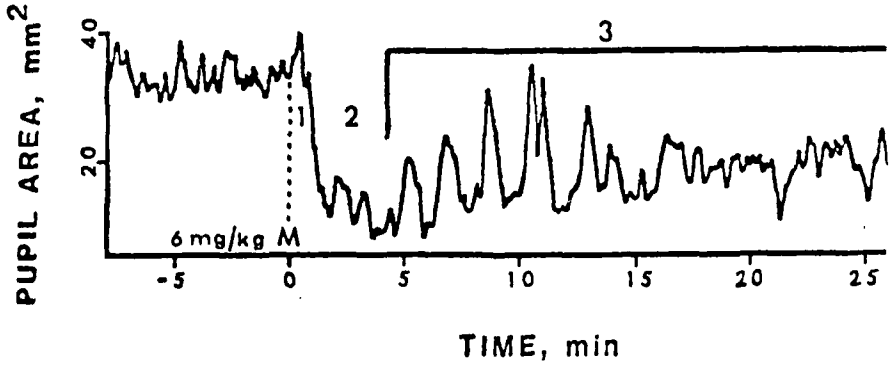

B
PUPIL AREA, mm²
40
20
1
2
3
6 mg/kg M
-5
0
5
10
15
20
25
TIME, min

Figure 2

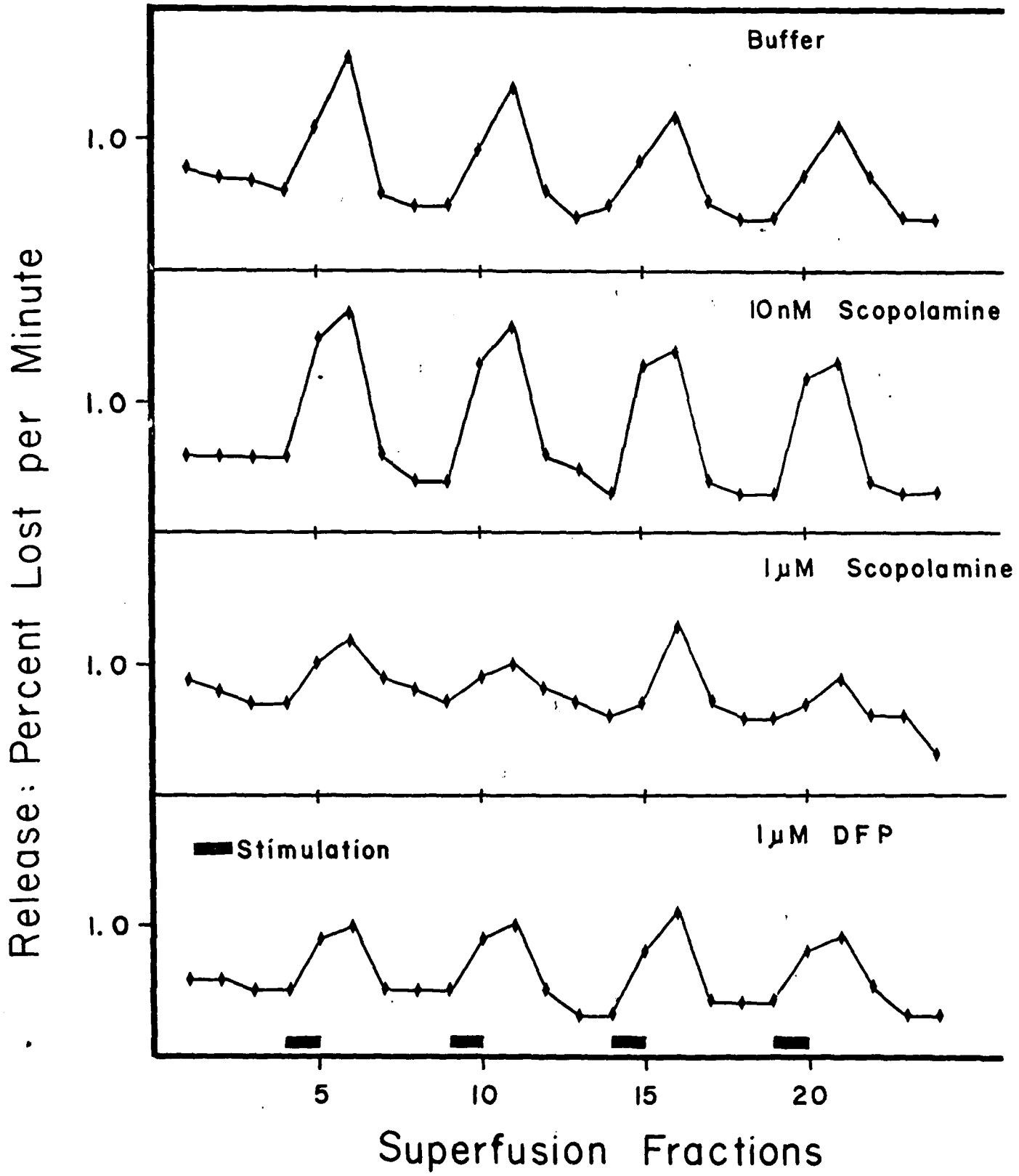

Buffer
10nM Scopolamine
1μM Scopolamine
1μM DFP
Stimulation
1.0
1.0
1.0
1.0
5
10
15
20
Release: Percent Lost per Minute
Superfusion Fractions

Figure 4

MULTIPLE EFFECTS OF DFP ON CHOLINERGIC SYNAPSES

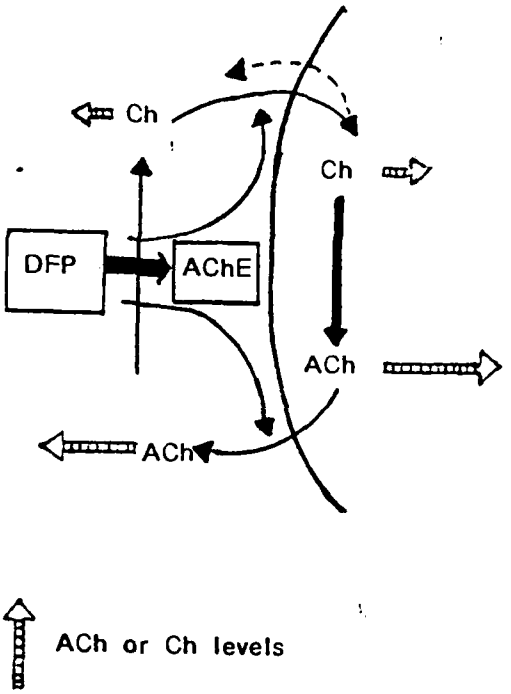


Figure 6

Amer. Soc. for Neurochemistry - Thirteenth Annual Meeting, Grossinger, New York
March 14-19, 1982

UPTAKE AND RELEASE IN CHOLINERGIC NERVE TERMINALS OF THE RAT IRIS.

Richardson, J.S., Matti, T.S.*, and Giacobini, E.
Pharmacol USASK Saskatoon SK S7N0W0 & Biobehav Sci U CONN Storrs CT 0626

Prior to an investigation of the acute and chronic effects of drugs inhibiting cholinesterase on the biochemical parameters of uptake, release and metabolism in cholinergic neurons innervating the eye, the uptake of choline and the release of acetylcholine were characterised in the isolated iris of the rat. The iris contains nerve endings whose cell bodies are located in autonomic ganglia. This makes the iris a good model for the study of nerve terminal function relatively free from contamination by cell body and glial effects.

In the choline uptake studies, the irises were each preincubated in .5 ml Elliot's B buffer for 5 min at 37° C, followed by the addition of .5 of carrier choline containing a tracer amount of tritiated choline. The incubation was continued for 5 min at 37°C. The uptake of choline by the rat iris is linear over 6 concentrations of choline ranging from 0.1 μM to 10 μM. The linear regression correlation coefficient is 0.99. The uptake of 1 μM choline by the iris is reduced by over 50% ($p < .001$) in low sodium buffer, and is blocked in a dose dependent manner by 0.01 μM (no effect) to 100 μM (32% reduction $p < .001$) ouabain. Compared to uptake at 37° C, choline uptake is reduced by 68% ($p < .001$) at 20° C, and by 85% ($p < .001$) at 0° C. In the release studies, the iris was prelabeled with 1 μM choline plus tracer tritiated choline, placed in a stimulation chamber and perfused with Elliot's B buffer at 37°C at a rate of 1 ml per min. Electrical stimulation of the iris by 20 mA, 5 msec, 100Hz nearly square waves, caused an almost 200% increase in the outflow of radioactivity. The identity of this radioactivity and the effects of drugs on uptake and release are being established.

(Supported by Grant AFOSR-81-0229 to E.G.)

UPTAKE AND RELEASE IN CHOLINERGIC NERVE TERMINALS OF THE RAT IRIS.

Dr J. Steven Richardson

Dept of Biobehavioral Sciences, Univ of Connecticut, Storrs, CT 06268
(On Leave from Univ of Saskatchewan)
203 486-2554

MECHANISMS OF CHOLINE UPTAKE AND ACETYLCHOLINE RELEASE IN PERIPHERAL CHOLINERGIC SYNAPSES.

Giacobini, E., Richardson, J.S. and Mattio, T.G., Laboratory of Neuropsychopharmacology, Department of Biobehavioral Sciences, University of Connecticut, Storrs, CT 06268, USA

A new procedure allowing to perform a multiple set of microanalyses of ACh (acetylcholine) metabolism and release, as well as of Ch (choline) uptake, has been applied to segments of single rat irises. The characteristics of the high and low affinity Ch uptake system which have been previously described by us for the developing and aging avian iris (Marchi et al., Dev. Neurosci. 2, 139, 1980 & Brain Res. 196, 423, 1980) have now been determined for the adult rat iris as well. As in the chicken, the rat iris exhibits two distinct Ch uptake systems. One component, a Na^+ dependent, temperature sensitive, high affinity system ($K_m = 1.37\ \mu M$) which is blocked by ouabain and hemicholinium, is most likely confined to cholinergic nerve terminals. A second component, probably localized in the iris muscle cells, is Na^+ independent and shows low affinity ($K_m = 433.3\ \mu M$). Only the high affinity component is reduced by μM concentrations of scopolamine and DFP. Electrical stimulation of the isolated iris by 20 mA, 5 msec 100 Hz nearly square waves causes a 200% increase in the outflow of radioactivity following incubation with (^{3}H)Ch in the presence of scopolamine. Scopolamine and DFP alter the release profile with 10 nM scopolamine increasing the evoked release, 1 μM scopolamine increasing spontaneous release, while 1 μM DFP reduces both the spontaneous and evoked release. These results are consistent with the existence of presynaptic muscarinic autoreceptors that control the release of ACh from the cholinergic terminals in the rat iris.

(Supported by Grant AFOSR-81-0229 to E.G.).

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Topic number & title: D -01 Acetylcholine

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EFFECTS OF DFP AND OTHER DRUGS ON CHOLINERGIC NERVE FUNCTION IN THE RAT IRIS. J.S. Richardson, T.G. Mattie*, M.L. Bernstein-Goral* and E. Giacobini. Lab. of Neuropsychopharmacology, Dept of Biobehavioral Sciences, Univ. of Connecticut, Storrs, CT 06268.

The analysis of acetylcholine (ACh) levels, metabolism and release, as well as the uptake of choline, were performed on segments of rat iris to investigate the mechanisms involved in the response of the iris to acute and chronic cholinesterase inhibition. At various times after the topical administration of DFP in sesame oil to the corneal surface, the rats were decapitated and the irises were removed. Pupil diameter was measured and ACh levels and cholinesterase activity were determined in each iris following extraction of remaining free DFP with chloroform. No changes were detected 1 min after the DFP, but by 5 min., pupil diameter was reduced by 50%, esterase activity was reduced by 90%, and ACh was increased by 6X. There were no further changes 30 min. after the DFP, but by 60 min., ACh had returned to control levels even though esterase activity was still inhibited by 90% and pupil diameter was still reduced by over 50%.

Choline is taken up by the rat iris by a low affinity process (K_M=106.6 μM) and by a high affinity active transport system (K_M=1.16 μM) that is temperature sensitive, sodium dependent, and is blocked in a dose dependent manner by hemicholinium in 10 μM or greater concentrations. Choline uptake is also reduced by millimolar concentrations of scopolamine and ouabain.

Electrical stimulation by 20 mA, 5 msec, 100Hz nearly square waves of isolated rat iris prelabled by incubation at 37C in Elliot's B buffer with tritiated choline, evokes a 1- to 2-fold increase in the release of tritium over the spontaneous release during pre-stimulation baseline. Scopolamine and DFP alter the release profile with 10 nM scopolamine increasing evoked release, 1 μM scopolamine increasing spontaneous release, and 1 μM DFP reducing both spontaneous and evoked release. These results are consistent with the existence of presynaptic muscarinic receptors that control the release of ACh from cholinergic nerve terminals in the rat iris.

Supported by Grant AFOSR-81-0229 to Dr Ezio Giacobini.

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